The University of South Bohemia in České Budějovice

Faculty of Science

Subcellular insight into cholesterol-mediated proliferation of the tick cell line

Master thesis

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České Budějovice 2022

Kropáčková, S., 2022: Subcellular insight into cholesterol-mediated proliferation of the tick cell line. Mgr. Thesis, in English. – 39 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation: Arthropods, including ticks, are not able to synthetise cholesterol *de novo*. However, cholesterol is an essential component of their cell membranes and serves as a precursor for the synthesis of steroid hormones. In this thesis, I looked at the biological function of exogenous cholesterol on the tick *Ixodes ricinus* cell line IRE/CTVM19. I verified the need for cholesterol supplementation in the growth medium for proper cell proliferation. Cellular proliferation was stimulated by cholesterol and not by other sterols (ergosterol, sitosterol). We further explored the two assumed aforementioned cholesterol cellular roles, but also investigated the role of cholesterol as a signal molecule: The effect of cholesterol sensing is described on the tick cell level, as well as on the level of the whole organism, using the *ex vivo* feeding system of ticks. This work provides novel insights into the cholesterol biology of ticks and tick cells, increasing our understanding of tick-host blood molecules' interaction.

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V Českých Budějovicích dne …… ……..

Sára Kropáčková

Acknowledgement (in Czech):

Děkuji svému školiteli Honzovi Pernerovi za jeho trpělivost, čas a odborné rady. Ráda bych poděkovala Petru Kopáčkovi za přijetí do jeho laboratoře, ve které mi dovolil učit se laboratorním metodám a umožnil mi pracovat na zajímavých experimentech. Dále bych ráda poděkovala členům laboratoře za výbornou atmosféru a ochotu mi kdykoliv poradit. Speciální poděkování patří Danielu Wüstnerovi a členům jeho týmu za rozšíření mých vědeckých obzorů. A velké díky také mé rodině, mému příteli a přátelům.

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List of abbreviations used

20-HE	20-hydroxyecdysone
27-OH-CTL	27-hydroxy-cholestatrienol
BSA	Bovine Serum Albumin
CTL	Cholestatrienol
Chol	Cholesterol
DHE	Dehydroergosterol
FBS	Fetal Bovine Serum
Hh	Hedgehog
FullS	Human Full Serum
LPDS	Human Lipoprotein Deficient Serum

1. Outline

The aim of the work was to describe why cholesterol is important for ticks, studied mainly through a cell line IRE/CTVM19 (Bell-Sakyi et al., 2012). For this thesis, we have outlined these questions:

a) Does cholesterol supplement in the media affect proliferation of tick cells?

b) What is the extent of sterol-specificity?

c) Does the exogenous availability of cholesterol affect the quantity of intracellular pool of cholesterol?

d) Does exogenous availability affect the subcellular distribution of cholesterol?

e) Can cholesterol be a ligand for a receptor or signal transduction pathway?

f) How translatable is cell-level research to a whole-organism level?

2. Literature review

2.1. Sterols across Tree of Life

Sterols are essential components of the metabolism of organisms across the tree of life, being involved in the regulation of key cellular and developmental processes. Sterols are generally found as components of biological membranes, where they regulate membrane fluidity, rigidity, permeability, act as membrane reinforcers (Ribeiro et al., 2007), or provide protection against oxidative stress (Galea and Brown 2009). Sterol compounds are also important substrates for hormone synthesis, such as the human body needs cholesterol to produce important steroid hormones, estrogen, and progesterone, and sitosterol is a precursor for brassinosteroids, hormones regulating plant growth (Lindsey et al., 2003).

There are several types of sterols, and a group of organisms usually share a specific type of sterol. For example, cholesterol is synthesised by vertebrates, ergosterol by fungi. Plants have a more complex sterol composition, most often containing stigmasterol or sitosterol. A special group of sterols is formed by hopanoids, sterols of primitive bacteria (Jing and Behmer, 2020).

Sterols are organic amphipathic molecules with a polar OH group on the A ring and the non-polar rest, with the main differences on the side chains of ring D (Figure 1). Cholesterol, sitosterol, and stigmasterol have the same structure in the ring core part of the molecule, ergosterol has also one more double bond added to the B ring. All the aforementioned sterols have branched-chain on the fourth ring (Fig. 1). Stigmasterol and sitosterol have compared to cholesterol additional alkyl groups in the chain part. Stigmasterol has also one more double bond in the chain part. Ergosterol has one more additional double bond in the chain part, where also an additional methyl group is present (Fig. 1).

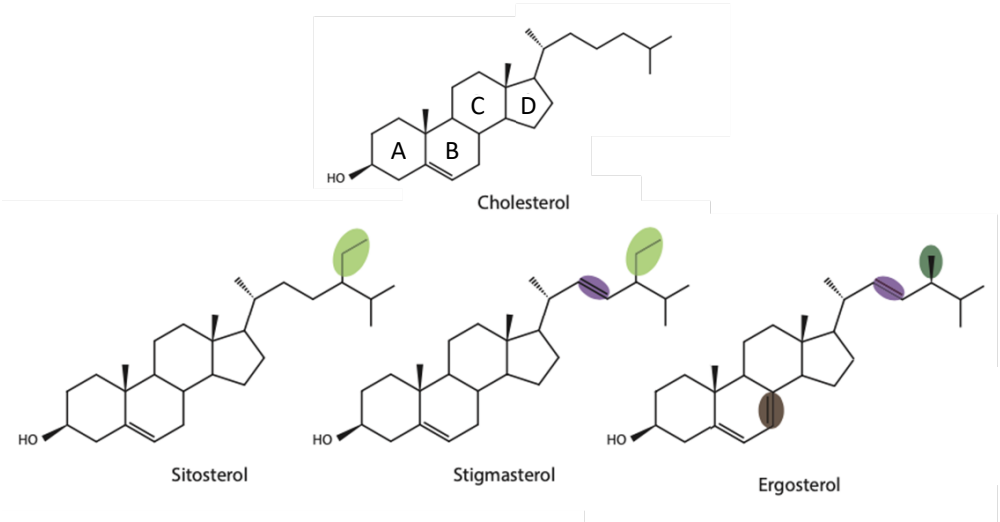


Figure 1. Structures of sterols, which are used by different organisms. Molecular structure of cholesterol, sitosterol, stigmasterol, and ergosterol with marked changes that differ from cholesterol, adapted from Jing and Behmer, 2020. The brown circle indicates changes in the number of double bonds in the ring core part of the molecule compared to cholesterol, the purple circle indicates changes in the number of double bonds in the chain part. Green circles indicate additional alkyl groups in the chain part of the molecule.

2.1.1. Sterols in membranes and cell protection

The plasma membrane that surrounds animal cells consists of a mixture of lipids and embedded proteins. Sterols act as a buffer guarding the right properties of membrane fluidity. Cholesterol adjusts the bilayer structure of biological membranes by changes in fluidity, thickness, compressibility, and water penetration. Sterols can also protect membranes against disruptive antimicrobial peptides (Mason et al., 2007). New findings also suggest that lipids have a regulatory role in the function of membrane proteins.

2.1.2. Membrane dynamics

Sterols are considered to strengthen membranes and allow their molecular arrangement. Membrane dynamics, a gentle equilibrium between too fluid and too rigid membrane, is present in every living cell. Sterols play a key role in the regulation of membrane dynamics, providing for example transfer across the bilayer. Cholesterol molecule aromatic part, fused four-ring system, support the feature of increasing the order of fluid phases. And conversely, the branched-chain has an important modulatory role (Dufourc, 2008). The presence of sterols may have increased the fluidity of eukaryotic membranes, which is an important step in increasing cell size (Dufourc, 2008). Evolution has led to adaptation to different types of sterols, but also to the synthesis of sterol complexes using other lipids (sphingolipids, phosphoinositides) present only in certain membranes (Mason et al., 2007).

2.1.3. Lipid rafts

Sterols are important for the creation of lipid rafts, fluidized membrane states, which are critical for many primary biological processes such as cytoskeleton reorganization, signal transduction, asymmetric growth, or cellular sorting (Simons and Ehehalt, 2002).

Lipid rafts, lipid microdomains, are rich in cholesterol and saturated chains lipids (for example sphingolipids). Rafts are held by hydrogen bonding and van der Waals interactions, relatively weak forces. They have a different protein composition, which promotes the development of membrane transport or signal transduction.

Lipid rafts, because of their small size and transient state, have never been directly observed in living cells. Despite conflicting reports from past research, cholesterol has become essential to the study of this lipid raft concept (Sezgin et al., 2015). Mainly because of cholesterol properties, fluidization of the ordered environment, arrangement of a disordered environment, and density fluctuations, which are dependent on cholesterol, and they may be nanoscale size, below the limit of detection by microscopy (Sezgin et al., 2015).

2.1.4. Hedgehog signalling pathway

The Hedgehog (Hh) signalling pathway transmits information to embryonic cells. It is crucial for proper cell differentiation and plays a key role in the regulation of all animals' development (Ingham et al., 2011). There are three Hh homologues in mammals, Desert Hh, Indian Hh, and Sonic Hh, all three equally important for embryonic development. It was found that cholesterol can be one of the triggers of the pathway, replacing Hh proteins (Luchetti et al., 2016).

The mechanism of Hh signalling pathway in insects is based on Patched protein inhibiting Smoothened protein (Fig.2). When extracellular Hh protein binds to Patched, it stops inhibiting Smoothened. Smoothened then continues the Hh pathway which ends with the transcription of special genes in the nucleus (Chen et al., 2004).

The Hh signalling pathway, important not only for embryonic development, was shown to affect the growth rate of *Drosophila* larvae, it can shift start of pupae and it regulates starvation resistance (Rodenfels et al., 2014). It was also shown that the Hh signalling pathway suppression causes suppression in midgut cell growth in silkworm *Bombyx mori* (Zhu et al., 2020).

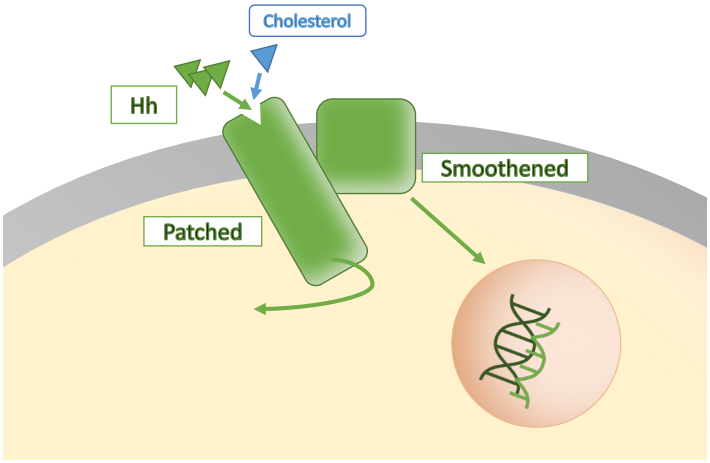


Figure 2. Scheme of the basic principle of the Hh signalling pathway. Hh proteins, or also cholesterol, bind to the Patched, Patched stops inhibition of Smoothened, and special genes in the nucleus are transcribed.

2.2. Sterol biosynthetic pathway and invertebrate auxotrophy

Cholesterol is biosynthesized is in all vertebrate cells in over 30 individual enzymic steps, the final molecule is biosynthesised from acetyl-CoA (Fig.3). Cholesterol is vital for development and cell growth, this is probably the reason why this pathway is highly conserved across species (Brown and Sharpe, 2016).

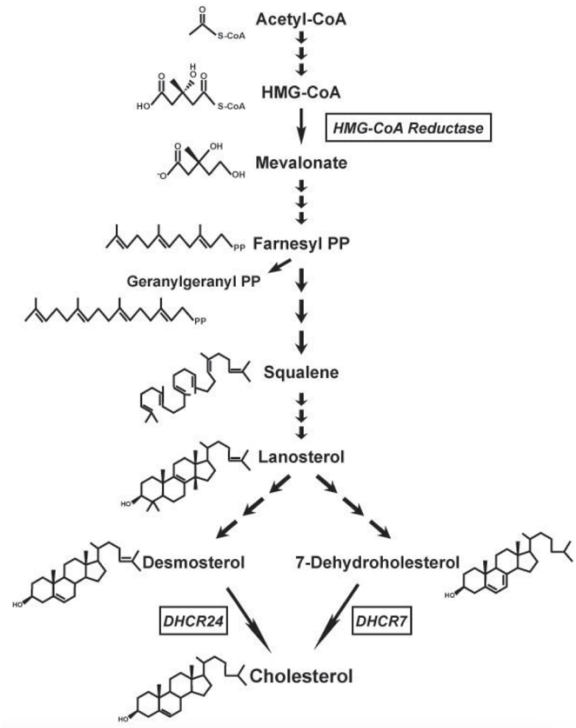


Figure 3. Description of the cholesterol synthesis pathway. Description of the vertebrate pathway with molecular structures of important intermediates (Cortes et al., 2014).

Most invertebrates, however, are not able to produce sterols over the sterol synthesis pathway and they are dependent on dietary sterol intake, they are sterol auxotrophs. The dietary sterol dependence in insects was described in the *Lucilia sericata* blowfly for the first time (Hobson, 1935). Sterol auxotrophy was later termed as the main proven difference in nutrient intake between most animals and insects (Clark and Bloch, 1959). This nutritional observation was later extended to more insect orders: Hemiptera, Coleoptera, Diptera, Orthoptera, Blattaria, Lepidoptera, and Hymenoptera (Svoboda, 1994).

Some organisms can use the sterol synthesis pathway at least partly. Nematodes are not able to make lanosterol precursors as the necessary genes are missing. But they can autotrophically produce farnesyl pyrophosphate, thereby being able to perform other biologically important reactions unrelated to sterol synthesis, for example, protein prenylation (Morck et al., 2009).

On the *Dermestes vulpinus* beetle was shown that cholesterol has two key roles, ensuring larval growth and development. The beetles were fed on a diet with a different amount of cholesterol or cholestanol, which differs in the only missing double bond. It was shown that cholesterol and cholestanol have no significant effect on the behaviour of the cell membrane in the presence of these molecules (Clayton, 1964). Cholestanol, which food has been supplemented with, has only a structural role, but cholesterol, added at least in small amounts, serves a metabolic role, the production of hormones affecting the growth and development of insects (Gilbert et al., 2002).

2.2.1. Sources of sterols for insects

Insects, sterol auxotrophs, are dependent on the sterol intake in their diet. Grasshoppers are well-studied animals, also from the point of view of sterols. A major part of the grasshoppers is feeder generalists, consuming a mixed plant diet. Cholesterol, cholestanol, and sitosterol are the only three sterols supporting growth and hatching (Behmer and Elias, 2000). Grasshoppers *Schistocerca americana* are not able to finish development while feeding on a diet when there is under 70% of suitable sterols compared to those that are unsuitable for them. Development can be disrupted even when there is enough of the suitable sterol, which alone would be enough for proper development, but combined with the unsuitable sterol. Grasshoppers have almost no tolerance for inappropriate types of sterols (Behmer and Elias, 1999).

Some invertebrates are able to process only a certain type of sterol. A member of the family Drosophilidae, *Drosophila pachea*, which reproduces in the stems of senita cactus, is a representative example of an insect which can utilize only a unique type of sterol coming from a cactus (Heed and Kircher, 1965). The Lepidoptera's larvae are mostly phytophagous, with some predaceous and ectoparasitic examples. Sitosterol and stigmasterol promote strong growth and development across tested species and some of the larvae of Lepidoptera, caterpillars that have grown in fungal environments, can utilize even ergosterol (Jing and Behmer, 2020).

There are known exceptions to animals that can take on a wider range of sterols. The need for dietary sterol has also been demonstrated in nematodes (Dutky et al., 1967). Nematode *Caenorhabditis elegans* living in soil, which is a cholesterol poor environment, have only limited access to cholesterol. But if exogenous cholesterol is removed, *C. elegans* can further survive, ergosterol or phytosterols can fulfil cholesterol demand (Hieb and Rothstein, 1968). Cholesterol is structurally similar to phytosterols, the only differences are in the side chain, which differs in length, saturation, and branching. It is known that *C. elegans* can modify the side chain of phytosterols to produce cholesterol from it (Shamsuzzama et al., 2020).

2.2.2. Intracellular and inter-tissue sterol transport in insect

Sterols obtained from a dietary source are transported to the tissues. It was better described in *Bombyx mori* silkworm, a phytophagous insect feeding on mulberry leaves, whose diet contains phytosterols and lacks cholesterol (Naito and Hamamura, 1961). Phytosterols from the midgut are absorbed through the epithelial cells and transformed into cholesterol by enzymatic reaction (Ciufo et al., 2011). Cholesterol is transferred from the midgut cell to hemolymph by lipophorin, a major insect lipoprotein (Chino et al., 1981). Insect tissues build in sterols from lipophorin carriers, thereby maintaining sterol homeostasis, whose disruption causes serious tissue damage (Rodríguez-Vázquez et al., 2015). As cholesterol is essential for various processes, the transmission of cholesterol is important as the regulation key of many pathways. It can be transferred between subcellular membranes by vesicular, non-vesicular, or membrane transport (Soccio and Breslow, 2004).

Sterol esters of fatty acids are stored in lipid droplets, dynamic and regulated organelles, which arise from ER (Martin and Parton, 2006). Mitochondria are also important in cholesterol processing pathways. Steroid hormones are synthetised *de novo* from

cholesterol. This pathway is catalysed by the enzyme P450SCC, the enzyme cleaving side chain, which is located in the inner mitochondrial membrane (Miller, 2007). Cholesterol processing is known in humans and some groups of insects, ticks still need to be further investigated.

Sterols can be radioactively or fluorescently labelled for easier trafficking in the cells. Fluorescent oxysterol analogue can be observed under a microscope, and it is an excellent new tool for sterol distribution studies. These microscopically detectable oxysterols must be chemically modified to gain these properties. Therefore, oxysterols with the smallest possible changes are used, usually with one or two added double bonds, to avoid excessive changes in biological activity (Maxfield and Wüstner, 2012). These intrinsically fluorescent sterols were used for example to study the distribution of sterols over time in living macrophages, adipocytes, and fibroblasts (Wüstner and Faergeman, 2008).

2.2.3. Cholesterol in mammalian cells

Cholesterol, yellow crystals when isolated, is the most abundant sterol circulating in the human body, in total there is 125-200 mg/dl of blood in a healthy person, and it makes up about 30 % of the cell membrane. It is produced by the liver and used is also for the synthesis of steroid hormones, cortisol, aldosterone and sex hormones, testosterone, progesterone, estrogens, and their derivatives (Hanukoglu, 1992).

Cholesterol, mostly in esterified form, can also be absorbed by the gut from food intake. When there is too much cholesterol in the diet the body can reduce its synthesis, which allows maintaining a balanced blood cholesterol level (Lecerf and Lorgeril, 2011). Cholesterol can interact with other membrane lipids, but also with specific proteins. Some proteins are directly involved in securing cholesterol homeostasis in the cell, for example, Niemann–Pick C1 protein (NPC1) (Nohturfft et al., 1998).

2.2.4. Cholesterol homeostasis and its transport in blood

Cholesterol is a water-insoluble molecule, and through plasma, it must be transported in the complex. Lipoproteins are particles, cholesteryl esters, and triglycerides, which make complexes for cholesterol transportation. There are five groups of lipoproteins, regarding their size and density of the protein to lipid content. Chylomicrons are the largest and least dense lipoproteins, transporting fatty acids to adipose tissue and muscles, and cholesterol

from the intestinal mucosa to the liver. Very low-density lipoproteins (VLDL) are smaller and denser than chylomicrons and distribute cholesteryl esters and triacylglycerols from the liver throughout the body. VLDLs are transformed into intermediate-density lipoproteins (IDL), and then finally into low-density lipoproteins (LDL) by lipoprotein lipases removing most of the fatty acids but sustaining cholesterol and cholesteryl ester concentrations. LDLs are the main cholesterol carriers in plasma, they can deliver to all tissues using receptor-enabled endocytosis. High-density lipoproteins (HDL) are the smallest and densest lipoproteins. HDLs contain mostly protein, phospholipids, cholesteryl esters, and cholesterol, and can also capture free cholesterol (Olson, 1998).

2.3. Ticks

The Ixodidae family of hard ticks are parasites from Arachnida class, Arthropoda phylum, occurring all over the world (Sonenshine, 1991). Ticks feed on the blood of their higher vertebrates' hosts and need blood as an exclusive source of protein and lipids from the larval stage of development (Sonenshine, 1991). During tick feeding, pathogens can be transmitted from one host to another (Jongejan and Uilenberg, 2004). This makes ticks important vectors of pathogens such as Lyme disease, tick-borne encephalitis, babesiosis, or ehrlichiosis (Sonenshine, 1991).

2.3.1. Castor bean tick (Ixodes ricinus)

The castor bean tick (*Ixodes ricinus*) is one of the common ectoparasites living in the Czech Republic. It feeds on the blood of 2-3 hosts, one host at each developmental stage (larva-nymph-adult), except eggs and adult males. Unlike a female, an adult male has a whole body covered by a solid cuticle. The adult female body is covered only one-third with the hard cuticle and it makes the rest of the body allowed to increase the volume while feeding.

The tick's largest organ is the gut, in which the host blood is processed (Sonenshine, 1991). Digestion takes place inside the intestinal cells (intracellularly), mainly haemoglobin and albumin are broken down by a combination of cysteine and aspartate proteases (Sojka et al., 2013; Sojka et al., 2016). The salivary glands are another important organ in the tick's body. It is a paired organ composed of secretory vesicles (acins) that produce saliva. Guts and salivary glands are included in the transmission of pathogens from the tick to the host.

Together with the host blood, ticks take in a lot of water, which must be disposed of. With water, ticks also pass pathogens, such as spirochetes *Borrelia afzelii*, causing Lyme disease. Tick gut is the decisive organ for *B. afzelii* spirochete transmission (Pospíšilová et al., 2019). Ticks are also dangerous carriers of the tick-borne encephalitis virus. Other, less common, tick-borne diseases include ehrlichiosis, caused by the bacteria *Anaplasma phagocytophilum*, babesiosis, and others. Especially *Babesia divergens* and *Babesia microti* can cause serious malaria-like diseases (Mosqueda et al., 2012).

2.3.2. Tick and blood-feeding

Hard tick females feed for roughly 7–8 days. First 6–7 days they feed slowly, then during rapid engorgement, which takes 12–24 hours, they reach about two-thirds of the total volume of the blood received (Sonenshine, 1991). Blood-feeding insects process blood in a short term of time and the pH neutral in the gut lumen (Briegel and Lea, 1975). In contrast, ticks feed slower and digest host blood in the gut epithelium in special acidic intracellular compartments (Grandjean and Aeschimann, 1973). Blood proteins are taken up by digestive gut cells using heterophagy (Sonenshine, 1991). Albumin is presumably taken up non-specifically by fluid-phase endocytosis while haemoglobin seems to be uptaken specifically by cell surface receptors (receptor-mediated endocytosis) (Lara et al., 2005).

Blood is a mix of plasma and cells that flow through the body of vertebrates, transferring essential substances to cells and organs, and removing waste substances from them. The main components of blood are plasma, red blood cells, white blood cells, and thrombocytes. Plasma, which makes more than half of the blood volume, consists mainly of water. Moreover, other substances are included in plasma, for example, glucose, hormones, proteins, mineral salts, vitamins, and lipids. All these substances are transferred to the tick body while feeding, including sterols.

2.3.3. Sterols and tick

Arthropods use molting to reach an adult form capable of reproducing. This process in insects and crustaceans is regulated by steroid hormones, ecdysteroids (Lafont et al., 2005). Insects can produce juvenile hormones by the mevalonate pathway (Bellés et al., 2005). Juvenile hormones affect embryonic development, metamorphosis, and pheromone production in insects (Nijhout, 1994). Ticks, *Ornithodoros moubata*, use ecdysone and 20-

hydroxyecdysone (20-HE) to control the molting (Germond et al., 1982). Adult tick females can convert ecdysteroids to their esters and store steroids in this form in eggs (Connat et al., 1984). Impregnated adult tick females produce higher ecdysteroids titers in the hemolymph than unfertilized females (Ogihara et al., 2007). This fact confirms that ecdysteroids are important for the tick reproduction cycle and it makes ticks, unable to synthesize cholesterol *de novo*, dependent on dietary cholesterol intake (Clark and Bloch, 1959).

Despite its importance for tick development and reproduction, the knowledge of lipid metabolism, inter-tissue transport, and sterol homeostasis has not yet been adequately studied (Sonenshine, 1992). The lipid role is well-described in insects, there it is a key component for energy storage and physiological processes, such as egg production or metamorphosis (Arrese et al., 2001). In the last years, ticks' lipid transfer was better described, a direct route of lipids from the fat body to the ovaries during the laying processes was discovered, and a *Rhipicephalus microplus* lipid carrier protein was described (Kluck et al., 2018). Given the importance of lipid processes in the tick body and the danger of ticks to humans, it is necessary to continue research in tick lipid transport to gain new targets for the control of these parasites.

2.3.4. Tick cell lines

Cell lines, that have been derived from vectors of dangerous pathogens, are important tools for finding new targets to fight these vectors (Bell-Sakyi et al., 2012). Continuous cultivation of tick cells lines was successful for the first time from tick *Rhipicephalus appendiculatus* (Varma et al., 1975). Tick cell lines, derived from molting nymphs or embryos, are phenotypically and genotypically heterogeneous (Bell-Sakyi et al., 2012). Attempts of tick cells cloning have been yet unsuccessful (Munderloh et al., 1994). Individual tick cell cultures can survive for long time periods, even years, with basic care of them (Bell-Sakyi et al., 2007). More than 60 continuous tick cell lines have now been set up, including IRE cell lines from *I. ricinus* (Bell-Sakyi et al., 2018).

The *I. ricinus* cell line IRE/CTVM19 grows properly in medium, which must be supplemented with 20% Fetal Bovine Serum (Bell-Sakyi et al., 2018). Fetal bovine serum (FBS) is the most used serum for cell culture supplementation, as it has few antibodies, a lot of growth factors, and the bovine serum albumin, which is a major source of protein in this serum. Serum from calves can be exchanged for human serum, in the case of tick cells, to

better mimic the feeding on its natural host. A serum can also be modified, such as deprived of lipoproteins, to study the effect on cell growth.

3. Aims of the work

- verification of the effect of cholesterol on IRE/19 cells proliferation
- comparison of the cholesterol function with other sterols
- comparison of the cholesterol amount and distribution in (non-)growing cells
- description of the function of cholesterol in cell proliferation
    - determination of the cholesterol essentiality for 20-HE synthesis in cells
    - examination of the cholesterol effect on the Hh signalling pathway

4. Materials and methods

4.1. Sterol conjugation on BSA

Sterols – cholesterol (Sigma-Aldrich, C8667), ergosterol (Sigma-Aldrich, E6510), sitosterol (Sigma-Aldrich, 43623) – 10 mg were dissolved in 1 ml Ethanol absolute (VWR Chemicals). Bovine Serum Albumin (BSA) (Sigma-Aldrich, A3059) (400 mg) was dissolved in 8 ml of M1 media (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$, 5mM Glucose.H_2O, 20 mM Hepes, pH 7.3). Solution of BSA in M1 media (8 ml) was mixed with 80 µl of sterol dissolved in ethanol, vortexed for 5 minutes, and incubated at room temperature for an hour. The final solution was filter-sterilized (filter 0.22 µm). The solution was mixed with cultivation media in a ratio 1:9. The final concentration of cholesterol in the media is 10 µg/ml (25.86 µM).

4.2. Ligand blot assessment of cholesterol coupling to BSA

Drops (2 µl) of diluted (15× – 50×) sera or cholesterol/BSA solution were loaded on the nitrocellulose membrane (Cytiva Whatman). Drops were allowed to dry for 10 minutes. The membrane was washed with Phosphate-buffered saline (PBS) + Tween (final concentration 0.05%). Autofluorescence was visualised with The ChemiDoc MP Imaging System (Bio-Rad) - Stain Free blot adjustment, exposure 0.1s. The membrane was then incubated with Filipin (50 µg/ml) (Sigma-Aldrich, F9765) for 20 minutes and washed with PBS + Tween. Bound cholesterol was visualised with ChemiDoc – Stain Free blot adjustment, exposure 0.1s. Finally, the membrane was incubated with Amido Black final concentration of 0.1% (Sigma-Aldrich) in 10% acetic acid for 15 minutes, then the membrane was distained with destaining solution (40% methanol, 10% acetic acid) and proteins were visualised with ChemiDoc – Colorimetry adjustment.

4.3. Media preparation

Two types of media (Tab. 1) were prepared: medium with Human full serum (**FullS**) and medium with Human Lipoprotein Deficient Serum (**LPDS**). The basis of the medium was Leibovitz L-15 medium (Biosera, LM-L1050), supplemented by Human serum (Sigma-Aldrich, H4522) or Human Lipoprotein Deficient Serum (Sigma-Aldrich, S5519), Tryptose Phosphate Broth (TPB) (Sigma-Aldrich, T8159), Antibiotic and Antimycotic (Antb) (Biosera XC-A4110) and L-glutamine (Glu) (Biowest, X0550).

Tab. 1: Comparison of the composition of the media used with percentages of individual components used in the final media.

FullS medium	LPDS medium
L-15 medium	L-15 medium
FullS 20 %	**LPDS 20 %**
TPB 10 %	TPB 10 %
Antb 1 %	Antb 1 %
Glu 1 %	Glu 1 %

4.4. Growth curves

IRE/CTVM19 cells (kindly provided by Dr. Lesley Bell-Sakyi from the University of Liverpool) were grown in two types of media, with the different cholesterol content. Cells were incubated in cultivation tubes (ThermoFisher, 156758) at 28°C. Media was supplemented with BSA, Linoleic Acid-Albumin from BSA (Sigma-Aldrich, L8384), Cholesterol-BSA (Chol-BSA), Sitosterol-BSA, Ergosterol-BSA, 20-hydroxyecdysone (Sigma-Aldrich, H5142) and Vismodegib (Selleckchem, S1082). The number of cells was measured in Bürker Counting Chamber using a microscope (10× objective) and Trypan Blue dye (ScienCell) in the final concentration of 0.2%. Cells were counted in technical quadruplicate.

4.5. Monitoring the ability of cells to take up different types of sterols using a fluorescence microscope

IRE/CTVM19 cells were incubated in FullS or LPDS media for 7 days. Then cells were transferred to glass-bottom dishes (ThermoFisher, 150680) coated with Poly-D-lysin (ThermoFisher, A3890401), and examined sterol was added to the media (final concentration = 10 μg/ml). Cells were incubated with sterols overnight. The next day cells were washed with M1 medium and observed under the microscope. Leica DMIRBE microscope with a 63 × 1.4 NA oil immersion objective (Leica Lasertechnik GmbH) with a Lambda SC smart shutter (Sutter Instrument Company) as illumination control were used for wide-field epifluorescence microscopy. Images were acquired with an Andor IxonEM blue EMCCD camera operated at -75 °C and driven by the Solis software supplied with the camera. Dehydroergosterol (DHE) (Avanti), Cholestatrienol (CTL), and 27-hydroxy-cholestatrienol (27-OH-CTL) (all three kindly provided in cooperation with an associate professor Daniel Wüstner from the Department of Biochemistry and Molecular Biology at the University of Southern Denmark, Odense) were imaged in the UV spectrum using a

specially designed filter cube obtained from Chroma Technology Corp. with 335-nm (20-nm bandpass) excitation filter, 365-nm dichromatic mirror, and 405-nm (40-nm bandpass) emission filter. Microscopy images were processed in ImageJ.

4.6. Sub-cellular localization of free cholesterol by confocal microscopy

Fully grown IRE/CTVM19 cells were transferred for microscopy to glass-bottom dishes coated with Poly-D-lysin. Cells were allowed to settle overnight at 28°C. Cathepsin-B activity in cells was indicated by Magic Red Cathepsin-B Assay Kit (ImmunoChemistry Technologies). The Magic Red substrate was used as described in the protocol included in the kit, shortly: Magic Red was dissolved in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich), then diluted 1:10 with distilled H_2O, 20 µl were used for one dish, followed by incubation in 28°C for 30 minutes. The Nile Red (ThermoFisher, N1142) staining was performed according to the protocol supplied in the package, shortly: 1 µl of Nile Red was mixed with 500 µl of Staining Buffer (included in the package) to generate the working Nile Red Staining Solution. 500 µl of Nile Red Staining Solution was used for one dish, incubated at 28°C for 30 minutes.

Cells were then fixed with 3% Paraformaldehyde (PFA) (Sigma-Aldrich) for 1 hour at room temperature. PFA was rinsed with PBS and quenched with 50mM Glycine in PBS for 20 sec. Cells were stained with Filipin (50 µg/mL in M1 media) for 2 hours at room temperature. Cells were washed with M1 medium and imaged in M1 media at room temperature using the Nikon Confocal microscope with the 100× NA 1.4 oil objective. Magic Red was observed with excitation at 592 nm and emission at 628 nm, Nile Red excitation at 550 nm and emission at 640 nm, Filipin with excitation at 360 nm and emission at 425 nm. Microscopy images were processed using ImageJ software.

4.7. Quantification of cholesterol in cells growing in different media

Cells were growing in FullS medium or starving in LPDS medium for 7 days. Cells in media were centrifuged (2500 × g, 10 min, 4°C), media were discarded, and pellets were washed with PBS. Cells pellets were diluted in 200 µl PBS and homogenised by freezing them in liquid nitrogen and thawing them in a heater (37°C), then centrifuged again (10000 × g, 10 min, 4°C). Supernatants were used for cholesterol measurement with Amplex™ Red Cholesterol Assay Kit by precisely following protocol from the kit. PBS was used as a negative control.

4.8. 20-hydroxyecdysone quantification

IRE/CTVM19 cells were grown in FullS and LPDS types of media with 20-hydroxyecdysone (20-HE) supplementation for 9 days. Then cells in the media were centrifuged (2500 × g, 10 min, 4°C), media were discarded, and pellets were washed with PBS. Quantitative analysis of 20-HE by HPLC was performed in collaboration with the Laboratory of Analytical Chemistry, Institute of Entomology.

4.9. cDNA synthesis and RT-qPCR comparison of Smoothened and Patched expression

Quantitative polymerase chain reaction, qPCR, was used to analyse the relative amount of transcripts in tick tissue samples. Template cDNA was used from a common cDNA library in the laboratory. Samples to the library were prepared by isolating total RNA from dissected tick gut using the Macherey-Nagel kit and 100 ng of the RNA was used for cDNA synthesis using oligo-dT primers according to Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

The cDNA was used as templates for subsequent quantitative expression analyses by RT-qPCR. qPCR reaction was prepared using Fast Start Universal SYBR Green Master Kit (Roche) with specific targeting primers (Tab. 2). To obtain the results of the primary analysis, measurements were performed on a LightCycler 480 instrument with Software release 1.5.0 SP4 (Roche), which recorded Cp values by the second maximum derivative method with arithmetic adjustment of the initial value to determine the crossing point of the specified fluorescence threshold (Cp). Secondary analysis was evaluated by delta-delta ct method (Excel, GraphPad Prism6). The relative level of the observed genes was normalized using the relative value of the reference gene *I. ricinus* elongation factor 1α (ef-1α).

Tab. 2: Overview of specific primers used to compare the expression of transcripts using qPCR.

Name of the target gene	Forward primer	Reverse primer
Smoothened	AGAACACCATGACGTTGGGG	TTTGCCGTGCTGACCTTCAT
Patched	CACCTCGACACCTCCGTAAC	TGTTCCTAGTGTCGCTCGTG

4.10. Membrane feeding of adult ticks

Membrane feeding of ticks was performed in a 6-well plate using feeding units with special membranes kindly provided by Dr. Perner and Bc. Hatalová. Unfed adult females and males of *I. ricinus* were used in the feeding units. Manually defibrinated bovine blood with gentamicin (5 mg/ml) and Vismodegib in various concentrations (0µM – 1,5mM) dissolved in DMSO was used for feeding. Bovine blood with gentamicin and DMSO was used as a negative control. The blood in the feeders was changed for fresh every 12 hours. After 7 days, photos of the feeding units were taken and after 14 days of feeding the adult females were removed and weighed.

5. Results

5.1. Exogenous cholesterol facilitates the proliferation of tick cells

Tick cells IRE/CTVM19, derived originally from tick *Ixodes ricinus*, were exposed to cholesterol-depleted conditions. We cultured them in media supplemented with Human Lipoprotein Deficient Serum (LPDS) and compared them to cells cultured in media supplemented with standard Human Full Serum (Full S). While cells reproduced normally when cultured with Full S supplement, cells did not proliferate when cultured with LPDS supplement (Fig. 4A).

To rescue the halt in cellular proliferation, we supplemented the LPDS-supplemented media with cholesterol conjugated to bovine serum albumin (BSA, Supplementary Fig. S1). The culture supplementation with BSA-cholesterol clearly rescued cellular proliferation of cells cultured with LPDS supplement, but it did not augment proliferation of cells cultured with Full S supplement (Fig. 4A). BSA-cholesterol was capable as a lipoprotein fraction to surrogate and maintain cellular proliferation after Full S to LPDS (Fig. 4A). Moreover, it managed to rescue growth after LPDS-mediated starvation for 5 days (Fig. 4B). These data document that cholesterol is the essential component of the deprived serum lipoprotein fraction. Unexpectedly, cellular proliferation was also rescued by linoleic acid-BSA, but no synergistic effect on rescued proliferation was observed for cholesterol and linoleic acid (Supplementary Fig. S2).

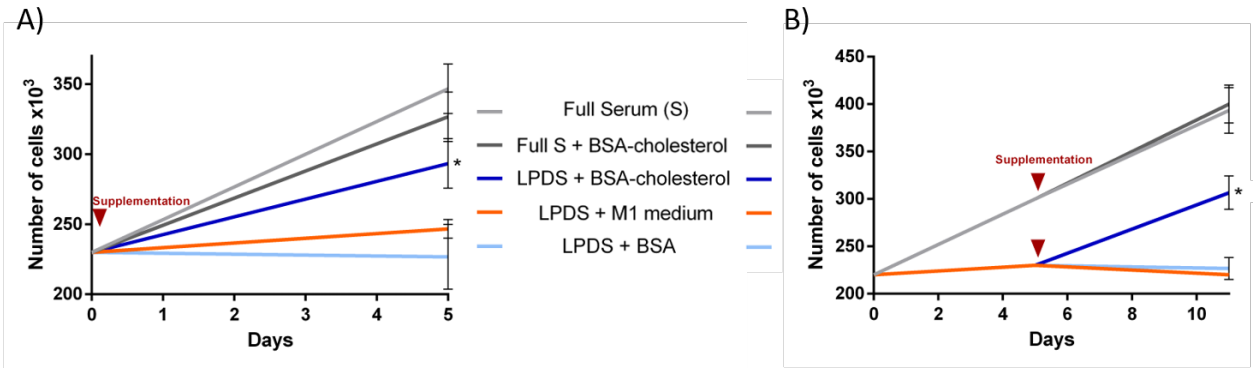


Figure 4. Analysis of cholesterol reliance of the tick *I. ricinus* cell line. Growth curves of IRE/CTVM19 cells grown in media with or without a serum lipoprotein fraction. Culture media contained Human Full Serum (S) or Human Lipoprotein Deficient Serum (LPDS). Cells were immediately co-cultured with exogenously supplemented components (A) or were allowed to starve with LPDS supplement and co-cultured after 5 days in the proliferation halt. Supplements were Cholesterol–BSA conjugate and unconjugated Bovine Serum Albumin (BSA). A number of cells in 1 ml of media is shown. Mean and SEM are shown, n = 3, * $p < 0.05$ T-test.

5.2. Tick cells display high specificity for cholesterol

We further looked at whether the ability to restore tick cellular proliferation is specific for cholesterol, or whether other sterols can substitute the serum lipoprotein fraction in media. Sitosterol (plant sterol) and ergosterol (sterol of fungi and protozoa) were tested, using again BSA as a conjugate vehicle. These sterols-BSA conjugates showed no capacity to rescue the cell proliferation of LPDS cultured cells (Fig. 5A).

We then examined if the sterols can be actually acquired into the cells or are acquired but cannot be further utilised. To test this, we used Cholestatrienol (CTL)-BSA and Dehydroergosterol (DHE)-BSA, as the fluorescent analogues of cholesterol and ergosterol, respectively, with distinct additional double bonds in the aromatic ring (Fig. 5B). While CTL was taken up by the cells, DHE was not. As a control for DHE uptake, we used human fibroblasts that indeed willingly take up DHE (Fig. 6A, B). These data indicate that the side chain and not the ring core is decisive for the cellular uptake of the distinct sterol. To inspect how fine the discrimination is, we used 27-hydroxy-cholestatrienol (27-OH-CTL)-BSA, a CTL analogue with one additional hydroxyl group at the terminus of side-chain of the molecule. Such a minute change, however, did not make a difference, as 27-OH-CTL was clearly taken up by tick cells (Fig. 6A, B) as well as rescued growth of tick cells cultured with LPDS supplement (Fig. 7). Our data suggest that the sterol side chain is the decisive structural fragment for cellular acquisition.

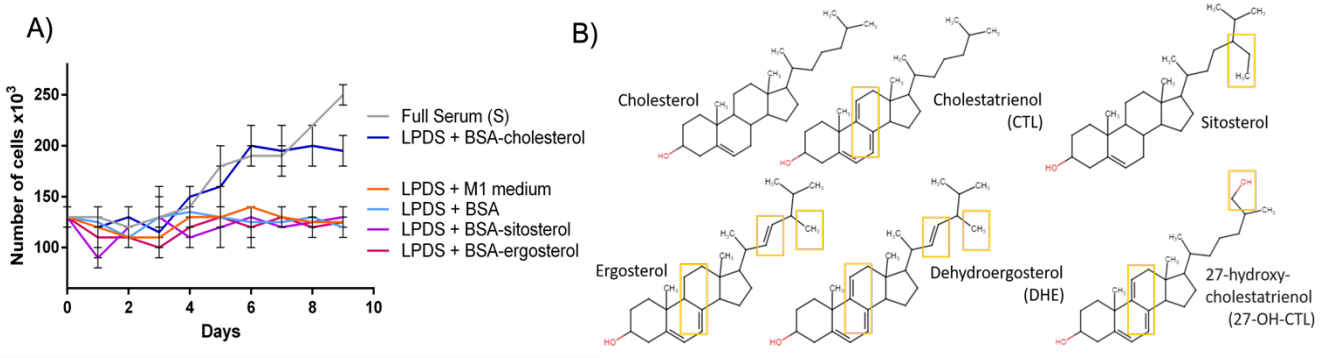


Figure 5. Comparison of different sterols. A) Growth curves of IRE/CTVM19 cells grown in media with different sterol supplements. Media contained human Full Serum (S) or human Lipoprotein Deficient Serum (LPDS). Cells were immediately rescued by exogenously supplemented components. Supplements were Cholesterol-BSA, Sitosterol-BSA, Ergosterol-BSA, or unconjugated Bovine Serum Albumin (BSA). A number of cells in 1 ml of media is shown. Mean and SEM are shown, n = 3. **B) Comparison of structures of used sterols.** Yellow boxes indicate differences from cholesterol.

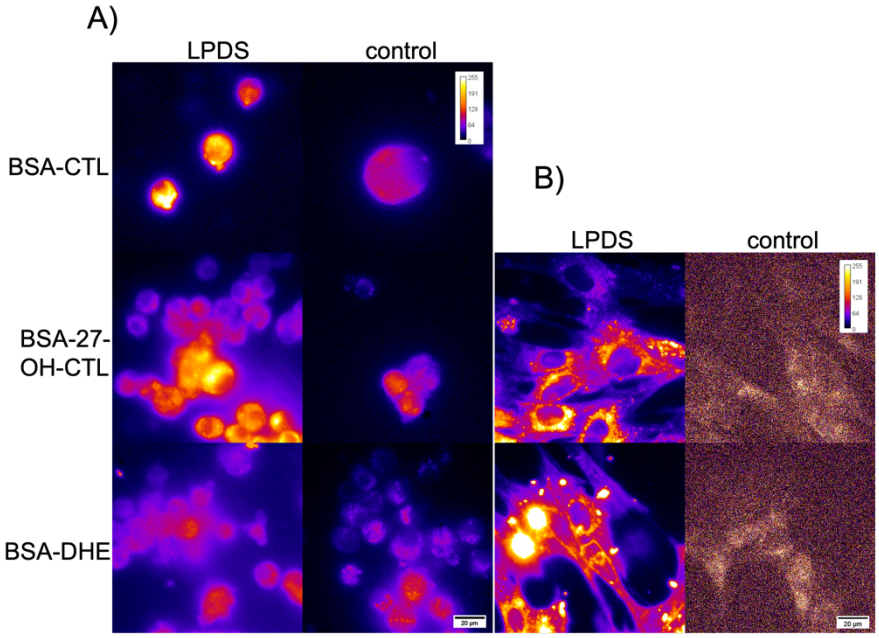


Figure 6. Visualisation of tick cells and fibroblasts uptake of different sterols using epi-fluorescence microscopy. A) IRE/CTVM19. B) Mammalian fibroblasts. Cells were incubated with Cholestatrienol (CTL) bound to Bovine Serum Albumin (BSA), 27-Hydroxycholestatrienol (27-OH-CTL) bound to BSA, or Dehydroergosterol (DHE) bound to BSA to assess the ability of cells to take up these sterols. Media contained human Lipoprotein Deficient Serum (LPDS). Cells incubated with medium only, with no sterol supplement, were used as a negative control. Cells were visualised under the fluorescence microscope showing the UV-range signal.

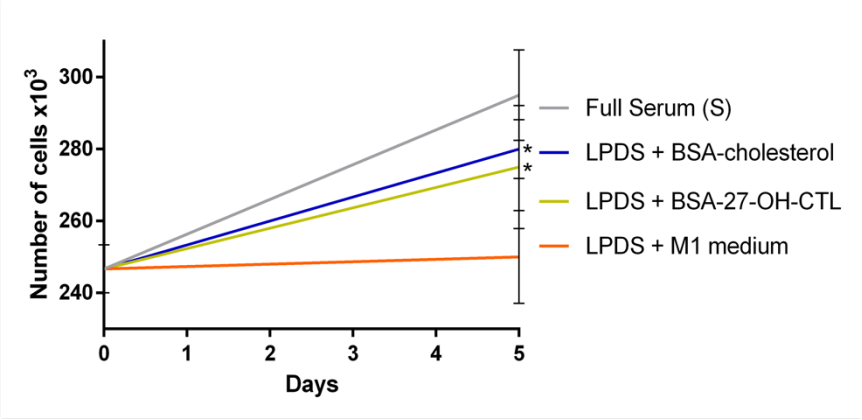


Figure 7. Growth curves of IRE/CTVM19 cells grown in media with different sterol supplements. Media contained human Full Serum (S) or human Lipoprotein Deficient Serum (LPDS). Cells were immediately rescued by exogenously supplemented components. Supplements were Cholesterol bound to BSA and 27-Hydroxycholestatrienol (27-OH-CTL) bound to BSA. A number of cells in 1 ml of media is shown. Mean and SEM are shown, n = 3, * $p < 0.05$ T test.

5.3. Exogenous cholesterol is not essential as a substrate for ecdysteroid biosynthesis

Ticks cells clearly display high specificity toward cholesterol molecular architecture, which triggers cellular proliferation. This indicates that cholesterol participates either as an enzymatic substrate or a receptor-ligand. To inspect the former hypothesis, we quantified 20-

hydroxyecdysone (20-HE), a final product of tick cholesterol metabolism (Lafont et al., 2005), in growing and starving cells (Fig. 8A). No 20-HE was identified in growing or starved cells cultured with LPDS supplement (Fig. 8B). When cells were cultured with LPDS supplement and 20-HE, 20-HE is abundantly present in the cells, indicating that the final product of tick cholesterol metabolism can only be acquired by tick cell line, and not produced endogenously. 20-HE is not decisive for cell proliferation because it was not detected in growing cells and its addition restores proliferation only partially.

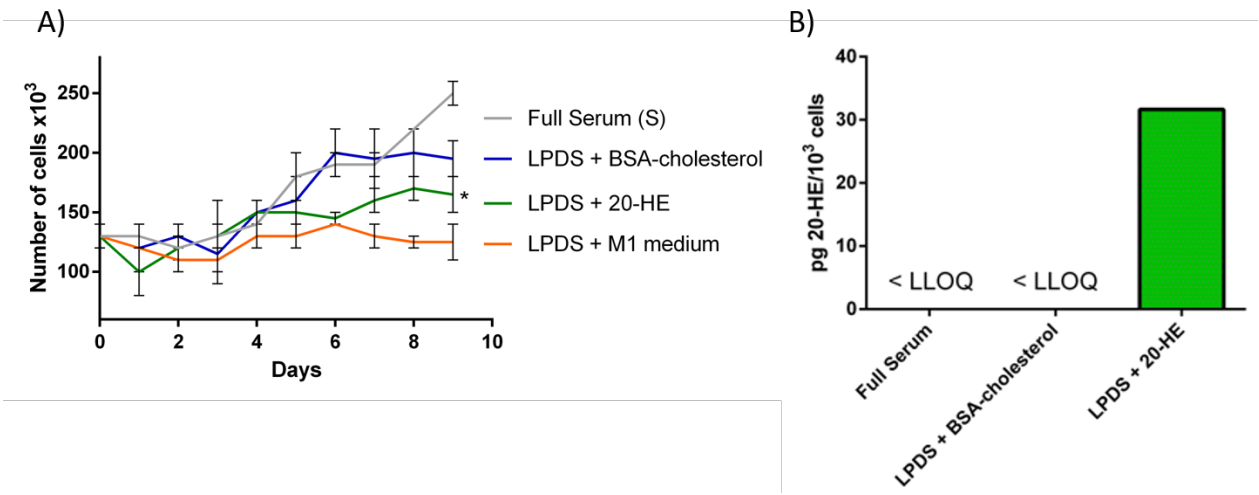


Figure 8. Analysis of 20-HE biosynthesis in the tick cell line. A) Growth curves of IRE/CTVM19 cells grown in media with cholesterol and 20-Hydroxyecdysone (20-HE) supplementation. Media contained human Full Serum (S) or human Lipoprotein Deficient Serum (LPDS). Cells were immediately rescued by exogenously supplemented components. Supplements: Cholesterol-BSA or 20-Hydroxyecdysone (20-HE). A number of cells in 1 ml of media is shown. Mean and SEM are shown, n = 3, * $p < 0.05$ T test. **B) Quantitative determination of 20-Hydroxyecdysone (20-HE) in IRE/CTVM19 cells.** Cells were harvested after 10 days of growth in media with the different cholesterol content. The lower limit of quantification is 0.28 fg per 1000 cells.

5.4. Tick cells store cholesterol in lipid droplets and plasma membrane irrespective of its exogenous availability

We studied the amount and distribution of cholesterol in the growing vs non-growing cells to understand the role of cholesterol in media. We assessed the quantity of intracellular cholesterol by two independent approaches: *i*) by binding filipin stain to free cholesterol in cells and quantification of emitting fluorescence (Fig. 9A) and *ii*) by enzymatic determination of intracellular cholesterol using Amplex Red based assay (Fig. 9B).

As the amount of quantified cholesterol in growing and non-growing cells by both approaches is comparable, we then looked at the intracellular distribution of cholesterol in the cells to detect differences (Fig. 10, 11). Using confocal microscopy, we identified Filipin

signal in lysosomes (co-localised with Magic Red), lipid droplets (co-localised with Nile Red), and plasma membranes of the cells. Nevertheless, no differences in cholesterol intracellular distribution between growing and non-growing cells were identified (Fig. 10, 11). Tick cells do not proliferate in the absence of external cholesterol even though they have the same amount of equally distributed cholesterol. We, therefore, speculate that cholesterol may not be essential for the proliferation of tick cell lines as an internal cellular component but rather as a signalling molecule mediating message at the interface of the cell and its environment.

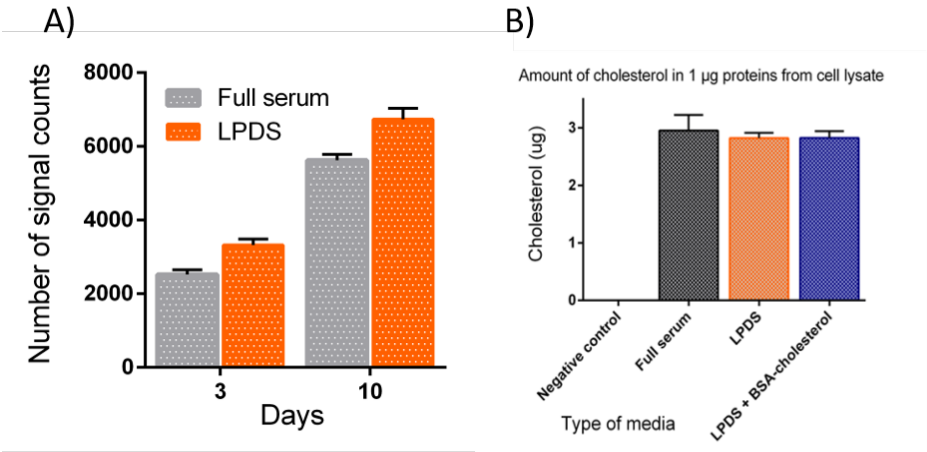


Figure 9: Cholesterol quantification. A) Quantification of filipin-sensitive free cholesterol signal using epi-fluorescence microscopy. A number of counts of Filipin signal from cells, which were growing in media with Full Serum and Lipoprotein Deficient Serum (LPDS). Cells were incubated in a determined types of media for 3 and 10 days. **B) Quantification of cholesterol in cells growing in different media.** The amount of cholesterol from cells, which were growing in media with Full Serum, Lipoprotein Deficient Serum (LPDS), and LPDS supplemented with cholesterol bound to Bovine Serum Albumin (BSA) was measured with using Amplex™ Red Cholesterol Assay Kit. Mastermix from the procedure with 1x Phosphate-buffered saline (PBS) was used as a negative control. Cells were growing/starving in each type of media for 7 days.

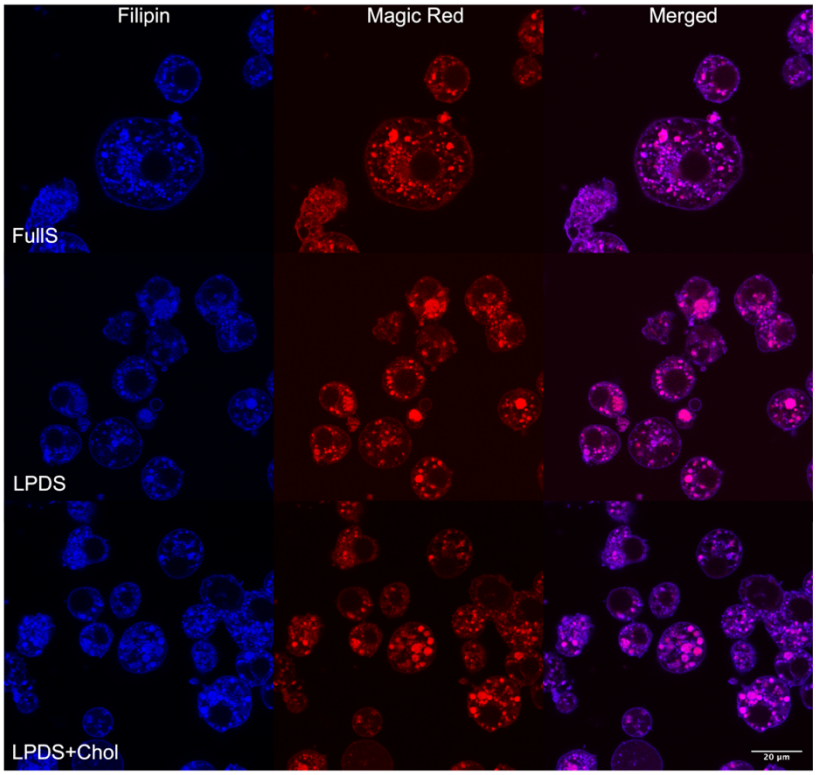


Figure 10. Sub-cellular localization of filipin-sensitive free cholesterol compared to lysosomal marker Magic Red in IRE/CTVM19 cells imaged by confocal microscopy. The merged channel shows the overlap of the two channels. IRE/CTVM19 cells were grown in media with Full Serum (FullS) and Lipoprotein Deficient Serum (LPDS), which was also supplemented with Cholesterol conjugated to bovine serum albumin (LPDS+Chol).

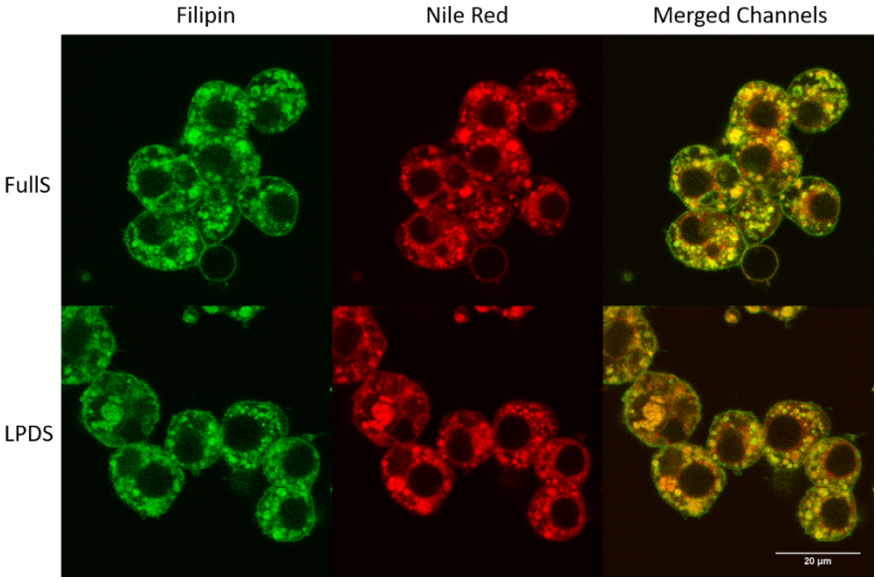


Figure 11. Sub-cellular localization of filipin-sensitive free cholesterol compared to lipid droplets marker Nile Red in IRE/CTVM19 cells imaged by confocal microscopy. The merged channel shows the overlap of the two channels. IRE/CTVM19 cells were grown in media with Full Serum (FullS) and Lipoprotein Deficient Serum (LPDS).

5.5. Cholesterol availability drives Hh signalling mediated cell proliferation

One of the proteins that bind cholesterol is Patched, a protein implicated in the signal transduction of Hh pathway. To test, whether we can blunt the signal transduction, we cultured cells in the full serum supplement, i.e., in the presence of cholesterol, and co-cultured them with Vismodegib, an inhibitor, which targets Smoothened, a protein downstream of Patched. If our hypothesis is correct, cells would cease proliferation even in the presence of exogenous cholesterol because they would be unable to recognise its presence, or in other words, will be unable to convey the message confirming Patched-cholesterol interaction. The growth curves clearly show that indeed Smoothened inhibition reduces cellular proliferation even in the Full Serum supplement, indicating that really the Patched-Smoothened part of the so-called Hh pathway seems to play a key role in facilitating cellular proliferation (Fig. 12). It is tempting to speculate that cholesterol plays a role as a master regulator representing exterior nutritional richness for tick cells.

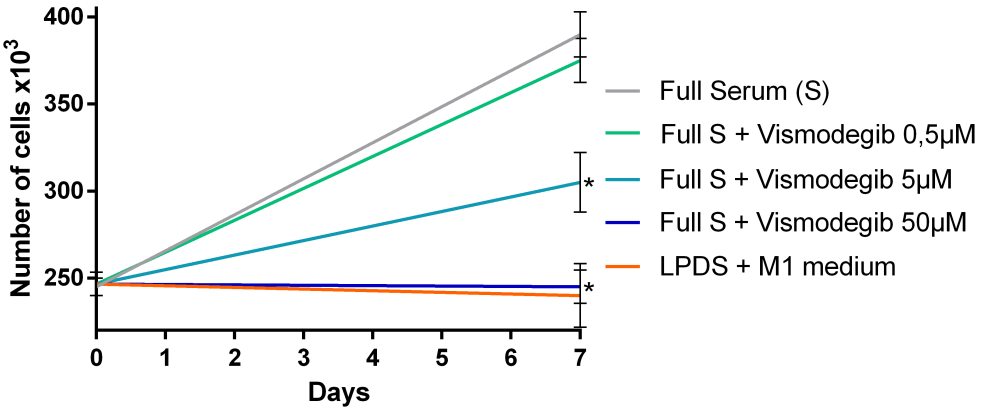


Figure 12. Growth curve of IRE/CTVM19 cells grown in FullS media with Vismodegib. Media contained Full Serum (S) from humans and Lipoprotein Deficient Serum (LPDS) from humans. Cells were incubated with Vismodegib in various concentrations. A number of cells in 1 ml of media are shown. Mean and SEM are shown, n = 3, * $p < 0.05$ T-test.

5.6. Comparison of Smoothened and Patched expression in tick tissues

To verify, whether we can translate our cell-level data to ticks, hypothesising that ticks would sense cholesterol through a Hh pathway as a "blood-representing" molecule, we checked the expression profile of Patched and Smoothened transcripts in tick midgut before and shortly after feeding initiation. We designed specific targeting primers according to orthologs from *Drosophila*. Sequence alignments show that tick Smoothened is quite different from insect forms, but it is identical in important parts of the Smoothened cysteine-

rich domain (Supplementary Fig. S3), tick Patched is similar to the form of insect (Supplementary Fig. S4). Gene expression tissue profiles of Smoothened and Patched show abundant expression of these with ongoing feeding mainly in tick ovaries (Fig. 13A, B). In contrast, intestinal expression is fairly low but seems to be relevant in the midgut of unfed ticks as would be expected for a dietary sensor (Fig. 13C, D).

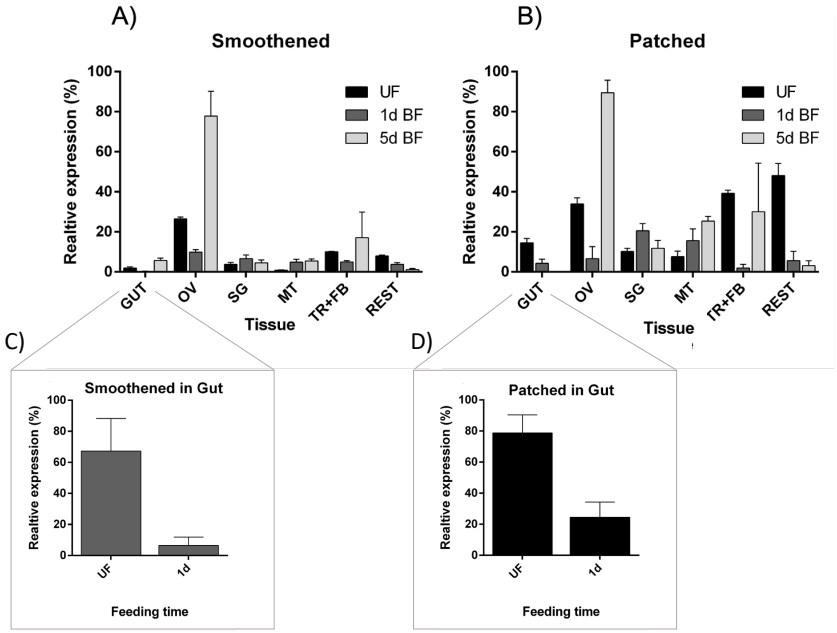


Figure 13: Gene expression profiles of Smoothened and Patched proteins in tick tissues. Unfed (UF) adult ticks of *I. ricinus* and adult ticks that were feeding on rabbits for one day (1d BF) or five days (5d BF) were used to detect whether genes are expressed in tissues. Tissues selected: guts (GUT), ovaries (OV), salivary glands (SG), Malpighi tubes (MT), tracheae, and fat bodies (TR+FB), and the rest of the tick body (REST). **A) Relative expression of Smoothened in tick tissues. B) Relative expression of Patched in tick tissues. C) Comparison of relative expression of Smoothened at the beginning of feeding in tick guts. D) Comparison of relative expression of Patched at the beginning of feeding in tick guts.**

5.7. Hh signalling pathway disruption limits tick feeding

To finally confirm whether ticks may sense blood meal through Hh pathway, we fed ticks *ex vivo* through a membrane feeding system, where ticks were served blood supplemented with the inhibitor Vismodegib. Indeed, we could see a deterring effect of the highest concentration of Vismodegib (Fig. 14A), indicating the inability of ticks to recognise blood meal and feed. Those ticks that initiated feeding struggled to feed and eventually to fully engorge even at single-digit µM concentration of Vismodegib supplementation (Fig. 14B). We tend to speculate that these data support a novel biological function for a protein

implicated in Hh pathway described for processes of embryogenesis and tissue & organismal development.

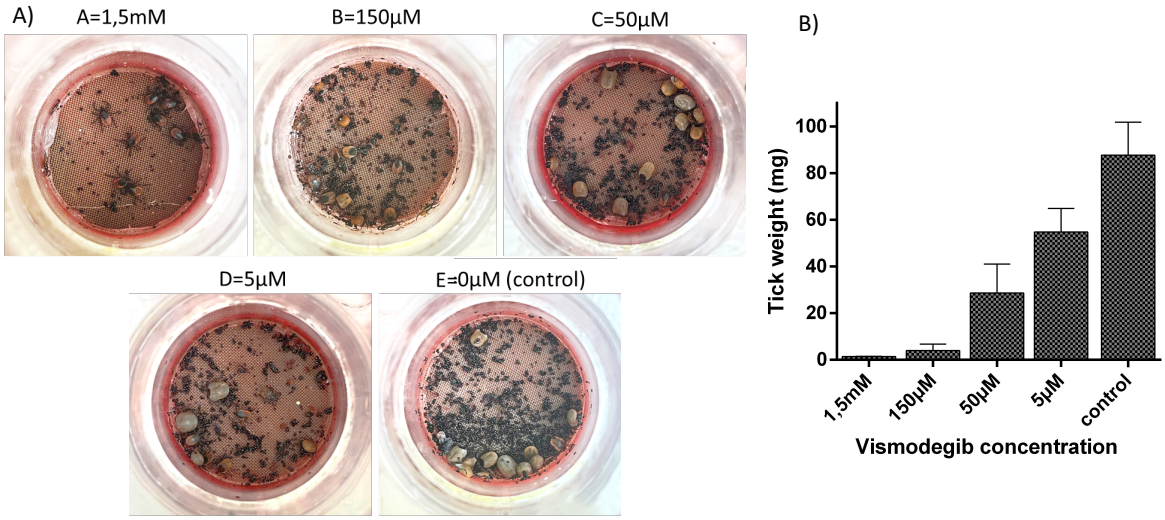


Figure 14. Analysis of Vismodegib impact on ticks feeding. A) Photos of the feeding unit with tick female adults with Vismodegib added in various concentrations in the blood. Bovine blood used for feeding contained Vismodegib dissolved in DMSO for experimental groups, or DMSO only for the control group. Photos were taken 7th day of feeding. **B) Graph with tick female adults' weights after 14 days of feeding.** Bovine blood used for feeding contained Vismodegib dissolved in DMSO in various concentrations for experimental groups, or DMSO only for the control group. Mean and SEM are shown, n = 6.

6. Discussion

Cholesterol is the cornerstone of the cell membranes of all animals. In addition to structural properties, it is essential for functional membrane dynamics, and signal transduction between cells, but also as a precursor for hormone synthesis and a signalling molecule of physiological pathways. Vertebrate cells synthesize cholesterol, arthropods, including ticks do not have this ability (Hobson, 1935). Ticks, ectoparasites that feed on host blood, which contains cholesterol, are thus dependent on dietary sterol intake.

The aim of the work was to describe the subcellular insight into the cholesterol-mediated proliferation of the tick cell line. Tick cells IRE/CTVM19 grow in a medium with Full human serum (FullS), but do not grow in the medium in which Lipoprotein deficient human serum (LPDS) is used. Cholesterol conjugated to bovine serum albumin (Chol-BSA) supplementation restores the proliferation of cells in a medium with LPDS and has no effect on cells growing in media with FullS. Cholesterol is hence needed for proper tick cell proliferation. Tick cells optimally grow in media with fetal bovine serum (FBS) supplementation (Bell-Sakyi et al., 2012). And cholesterol, contained also in the FBS, is probably the main component that cells in the LPDS medium lack. Even though the LPDS cells do not reproduce, they do not die. It seems like they are in an inhibited state awaiting internal cholesterol.

The cells grown in the media with LPDS supplement were clearly rescued by cholesterol supplementation (using BSA as a conjugation vehicle). Surprisingly, linoleic acid, originally thought of as an indifferent lipidic molecule to cholesterol, also showed a good capacity to restore cellular proliferation in the LPDS medium. No synergistic effect of cholesterol and linoleic acid supplementation on the restoration of proliferation was observed, suggesting they both target the same molecule/pathway. We speculate that linoleic acid can help trigger the pathway even at lower cholesterol concentrations. This process, when fatty acid is covalently bound to protein and causes benefits such as improvement of the hydrophobicity of proteins, was described before (Pepinsky et al., 1998). The use of fatty acid benefits is also associated with modification of the human Sonic Hh protein, influencing cell development (Ciepla et al., 2014). Tick cell growth is affected by either the addition of linoleic acid or in the absence of linoleic acid more cholesterol is needed to start the pathway. Our results show that linoleic acid and cholesterol do not have a synergistic effect. Like all groups, with cholesterol/linoleic acid/mix of both supplementations, grew at the

same rate as cells in the classical growth medium, we, therefore, assume that the cells reached maximum growth capacity and the synergistic effect would be observable only at a lower cholesterol concentration in the experiment.

We looked at whether the ability to restore tick cellular proliferation is specific to cholesterol, or whether other sterols have the same effect. Sitosterol and ergosterol were tested and showed no capacity to rescue the cell proliferation of LPDS cultured cells. We compared the intake of sterols of different structures, and we assume that the sterol side chain is the decisive structural fragment for cellular sterol acquisition. This is interesting because arthropods and nematodes if they do not have the option to obtain cholesterol directly from dietary intake, can uptake plant sterols and then convert them to cholesterol in their intestines (Clark and Bloch, 1959). This observation in ticks can be explained by the fact that they live from vertebrate blood, and they do not get in touch with any other type of sterol during their life. This is in line with the previous observation from grasshopper *Schistocerca americana*, as it has almost no tolerance for food intake of inappropriate types of sterols (Behmer and Elias, 1999).

Cells that have the same amount of cholesterol inside which is equally distributed may grow or not, depending on whether there is additional cholesterol in the surrounding medium. We think that cholesterol can be essential for cells for three different reasons: as a structural molecule, as a precursor for hormone synthesis, or as a signalling molecule for triggering a physiological pathway. Therefore, we explored whether cholesterol is important for IRE/CTVM19 growth as a precursor to the most common tick hormone 20-hydroxyecdysone, which is used to control tick molting (Germond et al., 1982). The chemical analysis did not show the presence of this hormone in proliferating cells and cell growth was not significantly affected by the addition of hormone to the medium. Therefore, cholesterol is not essential for cell growth in terms of hormone production.

The next step was to look at the distribution of cholesterol in the cells, which was found to be identical across the growing and non-growing groups. The resulting images from the co-localization experiments showed a significant signal overlap in the Filipin and Magic Red output. We believe that in this case, the Filipin signal infiltrated into the observation of lysosomal labelling, and so we focused on the second label used, Nile Red staining lipid droplets. We observed cholesterol in plasma membranes, which correlates with fact that cholesterol makes up about 30% of the cell membrane and is its basic building block. Cholesterol was also found in greater amounts of lipid droplets in cells. Lipid droplets are

formed as a normal cellular process and serve as fatty acid energy reserves (Greenspan et al., 1985). Their large numbers in both growing and starving tick cells could explain why cells in LPDS media do not die, they have a large supply of cholesterol for survival, and are probably just waiting for external cholesterol delivery for their growth.

We decided to directly measure and compare the amount of cholesterol in growing and starving cells. We assessed the quantity of intracellular cholesterol by two independent approaches, *i*) by binding filipin stain to free cholesterol in cells and quantification of emitting fluorescence (measures free cholesterol) and *ii*) by enzymatic determination of intracellular cholesterol using Amplex Red based assay (measures both free cholesterol and cholesteryl esters). Although there is a lot of cholesterol in the cell membrane, our results show almost 3 µg of cholesterol per 1 µg of cellular protein using Amplex Red assay. This does not seem like a plausible number and the method could also be supersaturated by the amount of cholesterol, so we decided to verify the amount of cholesterol with the help of chemical analysis. We are waiting for the results at the time of writing this thesis, and for now, we will be headed by the cholesterol ratios between growing and non-growing cells, which are more important for the work. Cells usually growing in FullS media have the same amount of cholesterol as cells starving, non-growing in LPDS media. This result was confirmed by both used approaches. Cholesterol has not only a structural role but serves also a metabolic role and the production of hormones (Gilbert et al., 2002). We believe that cholesterol in the external environment is necessary to trigger physiological pathways which affect cell proliferation and thus cells are more likely to reduce proliferation than to lower cholesterol in themselves.

The Hh signalling pathway transmits information to embryonic cells and is crucial for proper cell differentiation (Ingham et al., 2011). Cholesterol is one of the pathway triggers (Luchetti et al., 2016). We detected two proteins connected with this pathway, Smoothened and Patched, in the gut of the adult tick. Their expression decreases with the onset of feeding, we think that the pathway begins to activate at that time. In both Smoothened and Patched tissue expression profiles, the highest expression was observed in ovaries. The pathway is important for embryonic development, protein expression is higher here probably due to the development of eggs in the body of an adult female tick during feeding.

We mimicked the LPDS environment with a chemical inhibitor. The disturbance of the pathway was done using Vismodegib, which restricts Smoothened even though Patched

stopped inhibiting it, which would normally start the pathway. We observed the inability of the cells to grow even in their proper environment in FullS medium. By disrupting the Hh signalling pathway in cells, their growth is thus disrupted in the same way as when cholesterol is removed from the media. Therefore, we confirmed that cells without external cholesterol in the medium do not have an activated Hh pathway which causes them not to multiply. We believe that cells use the Hh pathway to signal whether the environment is suitable for proliferation in terms of cholesterol levels. Cholesterol is part of the host blood that ticks use as their only food source. We believe that a tick can use the Hh pathway as a signal for intestinal cells that feeding time has begun.

We transferred Hh inhibition method to an adult tick as a whole living organism using the system of artificial feeding with Hh pathway inhibitor in the blood meal. We found out that if this pathway is disrupted, it causes immediate trouble in tick to live. Ticks with increasing concentrations of inhibitor in their blood diet were becoming less and less able to reach the full size. The Hh signalling pathway, important not only for embryonic development, but was also shown to cause suppression in midgut cell growth in silkworm *Bombyx mori* (Zhu et al., 2020). And that pathway disruption causes brain, skeleton, musculature, gastrointestinal tract, and lungs problems in mouse development (Traiffort et al., 1998). Disruption of this pathway has probably the same effect on ticks.

To further confirm Hh pathway as a blood meal sensor in the tick midgut, we will further have to:

a) RNAi of Hh pathway components

b) Feed ticks LPDS sera + cholesterol supplement as an add-back

c) Establish read-out markers for Hh pathway activity: qPCR for Gli expression, WB against phosphorylated Gli protein activity...

These analyses will help us validate this novel biological output of the Hh pathway, specifically integrated into the biology of ticks and possibly other blood-feeding arthropods.

7. Conclusion

In this work subcellular insight into cholesterol-mediated proliferation of the tick cell line IRE/CTVM19 was studied. Cholesterol was described as a missing component of Lipoprotein Deficient Serum media necessary for proper cell proliferation. Subsequently, the inability of tick cells to utilize other sterols than cholesterol, more specifically ergosterol and sitosterol, was described. The same amount and distribution of cholesterol in both proliferating and starving non-proliferating cells were observed. It was further found that the cell line does not contain the hormone 20-hydroxyecdysone and that even its addition to the LPDS medium does not fully restore cell growth. We demonstrated essential proteins of the Hedgehog signalling pathway, Smoothened and Patched, in IRE/CTVM19 cells. Furthermore, we disrupted this pathway with an inhibitor and observed cessation of proliferation in the cells in the growing medium. In the end, we disrupted this pathway even in adult ticks feeding on the artificial feeding system. And we described the lower ability of the ticks to feed with increasing inhibitor concentration.

8. References

Arrese E.L., Canavoso L.E., Jouni Z.E., Pennington J.E., Tsuchida K., Wells M.A. (2001): Lipid storage and mobilization in insects: current status and future directions. Insect Biochemistry and Molecular Biology 31: 7–17.

Behmer S. T., Elias D. O. (1999): The nutritional significance of sterol metabolic constraints in the generalist grasshopper Schistocerca americana. Journal of Insect Physiology 45: 339–348.

Behmer S. T., Elias D. O. (2000): Sterol metabolic constraints as a factor contributing to the maintenance of diet mixing in grasshoppers (Orthoptera: Acrididae). Physiological and Biochemical Zoology 73: 219–230.

Behmer S. T., Grebenok R. J. (1998): Impact of sterols on life-history traits of a caterpillar. Physiological Entomology 23: 165–175.

Bell-Sakyi L., Darby A., Baylis M., Makepeace B.L. (2018): The Tick Cell Biobank: A global resource for in vitro research on ticks, other arthropods and the pathogens they transmit. Ticks and Tick-borne Diseases 9:1364–1371.

Bell-Sakyi L., Kohl A., Bente D.A., Fazakerley J.K. (2012): Tick cell lines for study of Crimean-Congo hemorrhagic fever virus and other arboviruses. Vector-Borne and Zoonotic Diseases 12: 769–781.

Bell-Sakyi L., Zweygarth E., Blouin E.F., Gould E.A., Jongejan F. (2007): Tick cell lines: tools for tick and tick-borne disease research. Trends in Parasitology 23: 450–457.

Bellés X., Martín D., Piulachs M.D. (2005): The mevalonate pathway and the synthesis of juvenile hormone in insects. Annual Review of Entomology 50: 181–199.

Briegel H., Lea A.O. (1975): Relationship between protein and proteolytic activity in the midgut of mosquitoes. Journal of Insect Physiology 21: 1597–1604.

Brown A.J., Sharpe L.J. (2016): Cholesterol synthesis. In Biochemistry of Lipids, Lipoproteins and Membranes. Boston: Elsevier.

Chen W., Ren X.R., Nelson C.D., Barak L.S., Chen J.K., Beachy P.A., de Sauvage F., Lefkowitz R.J. (2004): Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. Science 306: 2257–2260.

Chino H., Downer R.G.H., Wyatt G.R., Gilbert L.I. (1981): Lipophorins, a Major Class of Lipoproteins of Insect Haemolymph. Insect Biochemistry 11: 491.

Chitwood, D.J. (1999): Biochemistry and function of nematode steroids. Critical Reviews in Biochemistry and Molecular Biology 34: 273–284.

Ciepla P., Konitsiotis A.D., Serwa R.A., Masumoto N., Leong W.P., Dallman M.J., Magee A.I., Tate E.W. (2014): New chemical probes targeting cholesterylation of Sonic Hedgehog in human cells and zebrafish. Chemical Science 11: 4249–4259.

Ciufo L.F., Murray P.A., Thompson A., Rigden D.J., Rees H.H. (2011): Characterisation of a Desmosterol Reductase Involved in Phytosterol Dealkylation in the Silkworm, Bombyx Mori. PLoS ONE 6: e21316.

Clark A.J., Block K. (1959): The absence of sterol synthesis in insects. Journal of Biological Chemistry 10: 278–282.

Clayton R. B. (1964): The utilization of sterols by insects. Journal of Lipid Research 5: 3–19.

Connat J.L., Diehl P.A., Morici M. (1984): Metabolism of ecdysteroids during the vitellogenesis of the tick Ornithodoros moubata (Ixodoidea, Argasidae): Accumulation of apolar metabolites in the eggs. General and Comparative Endocrinology 56: 100–110.

Cortes V.A., Busso D., Maiz A., Arteaga A., Nervi F., Rigotti A. 2014): Physiological and pathological implications of cholesterol. Frontiers in Bioscience 19: 416–428.

De Smaele E., Ferretti E., Gulino A. (2010): Vismodegib, a small-molecule inhibitor of the hedgehog pathway for the treatment of advanced cancers. Current opinion in investigational drugs 11: 707–718.

Dufourc E.J. (2008): Sterols and membrane dynamics. Journal of Chemical Biology 4: 63–77.

Dutky S.R., Robbins W.E., Thompson J.V. (1967): The demonstration of sterols as requirements for the growth, development, and reproduction of the DD-136 nematode. Nematologica 13: 140–147.

Galea A.M., Brown A.J. (2009): Special relationship between sterols and oxygen: were sterols an adaptation to aerobic life? Free Radical Biology and Medicine 15: 880–889.

Germond J.E., Diehl P.A., Morici M. (1877): Correlations between integument structure and ecdysteroid titers in fifth-stage nymphs of the tick, Ornithodoros moubata. General and Comparative Endocrinology 46: 255–266.

Gilbert L. I., Rybcznski R., Warren J. T. (2002): Control and biochemical nature of the ecdysteroidogenic pathway. Annual Review of Entomology 47: 883–916.

Grandjean O., Aeschlimann A. (1973): Contribution to the study of digestion in ticks: histology and fine structure of the midgut ephithelium of Ornithodorus moubata, Murray (Ixodoidea, Argasidae). Acta Tropica 30: 193–212.

Greenspan P., Mayer E.P., Fowler S.D. (1985): Nile red: a selective fluorescent stain for intracellular lipid droplets. Journal of Cell Biology 3: 965–973.

Hanukoglu I. (1992): Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. The Journal of Steroid Biochemistry and Molecular Biology 43: 779-804.

Heed W. B., Kircher H. W. (1965): Unique sterol in the ecology and nutrition of Drosophila pachea. Science 149: 758–761.

Hieb W.F., Rothstein M. (1968): Sterol requirement for reproduction of a free-living nematode. Science 160: 778–780.

Hobson R.P. (1935): On a fat-soluble growth factor required by blow-fly larvae: Distribution and properties. Biochemical Journal 29: 1292-1296.

Ingham P.W., Nakano Y., Seger C. (2011): Mechanisms and functions of Hedgehog signaling across the metazoa. Nature Reviews Genetics 12: 393–406.

Jing X., Behmer S.T. (2020): Insect Sterol Nutrition: Physiological Mechanisms, Ecology, and Applications. Annual Review of Entomology 65: 251–271.

Jongejan F., Uilenberg G. (2004): The global importance of ticks. Parasitology 129: 3–14.

Kluck G.E.G., Silva Cardoso L., De Cicco N.N.T., Lima M.S., Folly E., Atella G.C. (2018): A new lipid carrier protein in the cattle tick Rhipicephalus microplus. Ticks and Tick-borne Diseases 9: 850–859.

Lafont R., Dauphin-Villemant C., Warren J.T., Rees H. (2005): Ecdysteroid Chemistry and Biochemistry. Oxford: Elsevier.

Lara F.A., Lins U., Bechara G.H., Oliveira P.L. (2005): Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick Boophilus microplus. The Journal of Experimental Biology 16: 3093–3101.

Lecerf J.M., de Lorgeril M. (2011): Dietary cholesterol: from physiology to cardiovascular risk. The British Journal of Nutrition 106: 6–14.

Lindsey K., Pullen M. L., Topping, J. F. (2003): Importance of plant sterols in pattern formation and hormone signaling. Trends in Plant Science 8: 521–525.

Luchetti G., Sircar R., Kong J.H., Nachtergaele S., Sagner A., Byrne E.F., Covey D.F., Siebold C., Rohatgi R. (2016): Cholesterol activates the G-protein coupled receptor Smoothened to promote Hedgehog signaling. Elife: e20304.

Martin S., Parton R. G. (2006): Lipid droplets: a unified view of a dynamic organelle. Nature Reviews Molecular Cell Biology 7: 373–378.

Mason A.J., Marquette A., Bechinger B. (2007): Zwitterionic phospholipids and sterols modulate antimicrobial peptide-induced membrane destabilization. Biophysical Journal 93: 4289–4299.

Maxfield F.R., Wüstner D. (2012): Analysis of cholesterol trafficking with fluorescent probes. Methods in Cell Biology: 108: 367–393.

Miller W. L. (2007): Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. Biochimica et Biophysica Acta 1771: 663–676.

Morck C., Olsen L., Kurth C., Persson A., Storm N.J., Svensson E., Jansson J.O., Hellqvist M., Enejder A., Faergeman N.J. (2009): Statins inhibit protein lipidation and induce the unfolded protein response in the non-sterol producing nematode Caenorhabditis elegans. Proceedings of the National Academy of Sciences 106: 18285–18290.

Mosqueda J., Olvera-Ramirez A., Aguilar-Tipacamu G., Canto G. J. (2012): Current Advances in Detection and Treatment of Babesiosis. Current Medicinal Chemistry 19: 1504–1518.

Munderloh U.G., Liu Y., Wang M., Chen C., Kurtti T.J. (1994): Establishment, maintenance and description of cell lines from the tick Ixodes scapularis. Journal of Parasitology 80: 533–543.

Naito K., Hamamura Y. (1961): Studies on the Micro Constituent in Mulberry Leaves Part VII. Isolation of Rutin and Quercetin from Mulberry Leaves. Agricultural Chemical Society of Japan 35: 848–850.

Nes W. D. (1987): Metabolism and function of sterols in Phytophthora cactorum. Journal of the American Oil Chemists' Society 64: 634–634.

Nijhout H.F. (1994): Insect Hormones. Princeton: Princeton University Press.

Nohturfft A., Brown M. S., Goldstein J. L. (1998): Sterols regulate processing of carbohydrate chains of wild- type SREBP cleavage-activating protein (SCAP), but not sterol-resistant mutants Y298C or D443N. Proceedings of the National Academy of Sciences 95: 12848–12853.

Ogihara K., Horigane M., Nakajima Y., Moribayashi A., Taylor D. (2007): Ecdysteroid hormone titer and its relationship to vitellogenesis in the soft tick, Ornithodoros moubata (Acari: Argasidae). General and Comparative Endocrinology 150: 371–380.

Olson R.E. (1998): Discovery of the lipoproteins, the role in fat transport and their significance as risk factor. Journal of Nutrition 128: 439–443.

Pepinsky R.B., Zeng C., Wen D., Rayhorn P., Baker D.P., Williams K.P., Bixler S.A., Ambrose C.M., Garber E.A., Miatkowski K., Taylor F.R., Wang E.A., Galdes A. (1998): Identification of a palmitic acid-modified form of human Sonic hedgehog. Journal of Biological Chemistry 22: 14037–14045

Perner J., Provazník J., Schrenková J., Urbanová V., Ribeiro J.M., Kopáček P. (2016): RNA-seq analyses of the midgut from blood- and serum-fed Ixodes ricinus ticks. Scientific Reports 6: 1–18.

Pospíšilová T., Urbanová V., Hes O., Kopáček P., Hajdušek O., Šíma R. (2019): Tracking of Borrelia afzelii transmission from infected Ixodes ricinus nymphs to mice. Infection and Immunity 87: e00896.

Ribeiro N., Streiff S., Heissler D., Elhabiri M., Albrecht-Gary A.M., Atsumi M., Gotoh M., Desaubry L., Nakatani Y., Ourisson G. (2007): Reinforcing effect of bi- and tri-cyclopolyprenols on ‘primitive’ membranes made of polyprenyl phosphates. Tetrahedron 63: 3395–3407.

Rodenfels J., Lavrynenko O., Ayciriex S., Sampaio J.L., Carvalho M., Shevchenko A., Eaton S. (2014): Production of systemically circulating Hedgehog by the intestine couples nutrition to growth and development. Genes & Development 28: 2636–2651.

Rodríguez-Vázquez M., Vaquero D., Parra-Peralbo E., Mejía-Morales J.E., Culi, J. (2015): Drosophila Lipophorin Receptors Recruit the Lipoprotein LTP to the Plasma Membrane to Mediate Lipid Uptake. PLoS Genetics 11: e1005356.

Sezgin E., Can F.B., Schneider F., Clausen M.P., Galiani S., Stanly T.A., Waithe D., Colaco A., Honigmann A., Wüstner D., Platt F., Eggeling C. (2016): A comparative study on fluorescent cholesterol analogs as versatile cellular reporters. Journal of Lipid Research 57: 299–309.

Shamsuzzama, Lebedev R., Trabelcy B., Langier Concalves I., Gerchman Y., Sapir A. (2020): Metabolic reconfiguration in C. elegans suggests a pathway for widespread sterol auxotrophy in the animal kingdom. Current Biology 30: 3031–3038.

Simons K., Ehehalt R. (2002): Cholesterol, lipid rafts, and disease. Journal of Clinical Investigation 110: 597–603.

Soccio R. E., Breslow J. L. (2004): Intracellular cholesterol transport. Arteriosclerosis, Thrombosis, and Vascular Biology 24: 1150–1160.

Sojka D., Franta Z., Horn M., Caffrey C.R., Mareš M., Kopáček P. (2013): New insights into the machinery of blood digestion by ticks. Trends in Parasitology 29: 276–285.

Sojka D., Pytelková J., Perner J., Horn M., Konvičková J., Schrenková J., Mareš M., Kopáček P. (2016): Multienzyme degradation of host serum albumin in ticks. Ticks and Tick-borne Diseases 7: 604–613.

Sonenshine D.E. (1991): Biology of Ticks. New York: Oxford university Press.

Svoboda J.A. (1994): Steroid metabolism as a target for insect control. Biochemical Society Transactions 22: 635–641.

Traiffort E., Charytoniuk D.A., Faure H., Ruat M. (1998): Regional distribution of Sonic Hedgehog, patched, and smoothened mRNA in the adult rat brain. Journal of Neurochemistry 70: 1327–1330.

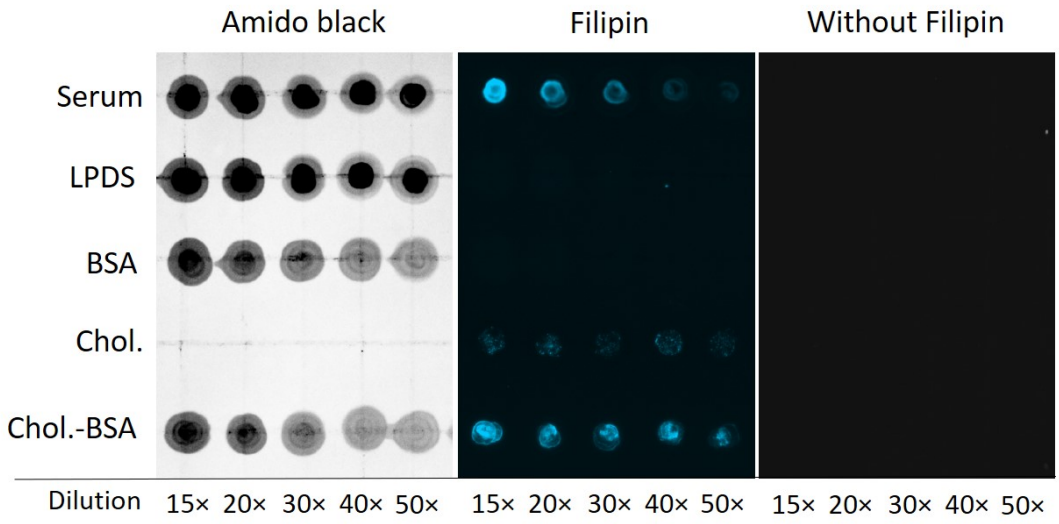
Varma M.G., Pudney M., Leake C.J. (1975): The establishment of three cell lines from the tick Rhipicephalus appendiculatus (Acari: Ixodidae) and their infection with some arboviruses. Journal of Medical Entomology11: 698–706.

Wüstner D., Faergeman N.J. (2008): Spatiotemporal analysis of endocytosis and membrane distribution of fluorescent sterols in living cells. Histochemistry and Cell Biology 130: 891–908.

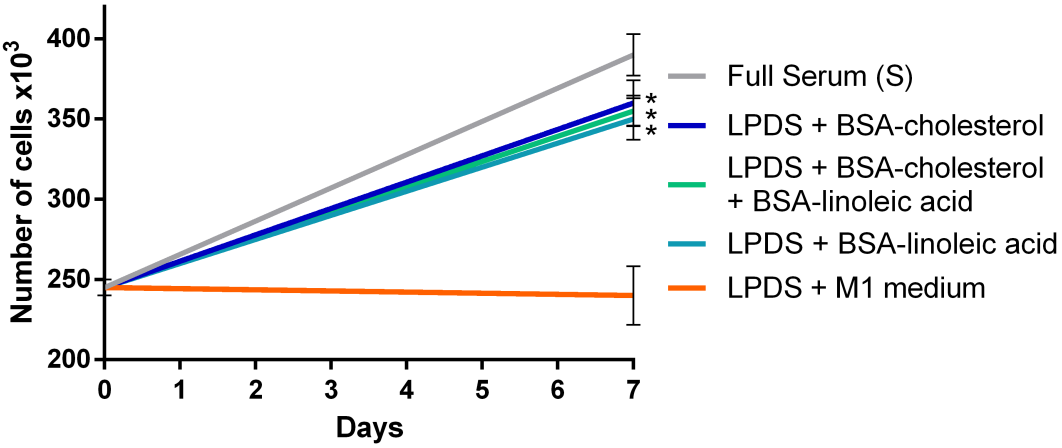
Zhu J., Khalil S.M., Mitchell R.D., Bissinger B.W., Egekwu N., Sonenshine D.E., Roe R.M. (2016): Mevalonate-Farnesal Biosynthesis in Ticks: Comparative Synganglion Transcriptomics and a New Perspective. PLoS One 11: e0141084.

Zhu S.Q., Zhang Y.J., Abbas M.N., Hao X.W., Zhao Y.Z., Liang H.H., Cui H.J., Yang L.Q. (2020): Hedgehog promotes cell proliferation in the midgut of silkworm, Bombyx mori. Journal of Insect Science 27: 697–707.

9. Supplement



Supplementary Figure S1. Ligand blot assessment of cholesterol coupling to BSA. The efficiency of conjugation of sterols on BSA was assessed by a ligand blot. Proteins in Serum, Lipoprotein Deficient Serum (LPDS), Bovine Serum Albumin (BSA), Cholesterol (Chol.), and Cholesterol bound to BSA (Chol.-BSA) were stained using the Amido black stain (left image). Drops in the first column contain 15× diluted sera or 6.7 µg of BSA (physiological value, the same concentration used for sterol binding). Cholesterol was stained with filipin (middle image), and the absence of autofluorescence was verified (right image). Samples were applied in 2µl drops.



Supplementary Figure S2. Growth curves of IRE/CTVM19 cells with single or multiple supplementation components. Different types of culture supplements were used. Media contained human Full Serum or human Lipoprotein Deficient Serum (LPDS). Media was also supplemented with Cholesterol - BSA conjugate or Linoleic acid conjugated to BSA. A number of cells in 1 ml of media is shown. Mean and SEM are shown, n = 3, * $p < 0.05$ T-test.

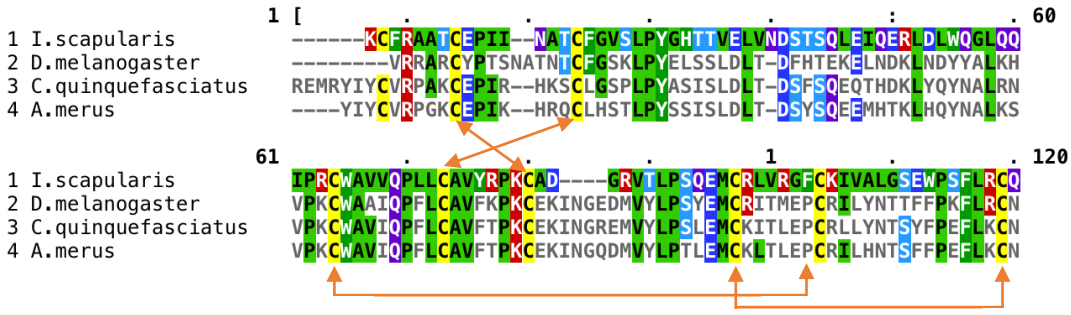


Figure S3. Sequence alignment of the Smoothened cysteine-rich domain. Smoothened has an extracellular cysteine-rich domain (CRD), necessary for its function in Hh signalling. Primary sequence alignment of the Smoothened CRD from Ixodes scapularis (XP_040063817.1), Drosophila melangoster (NP_523443.1), Culex quinquefasciatus (EDS37618.1) and Anopheles merus (XP_041779306.1). The disulphide bond pattern for the Smo CRD is shown in orange lines. The sequence alignment was generated using Clustal Omega.

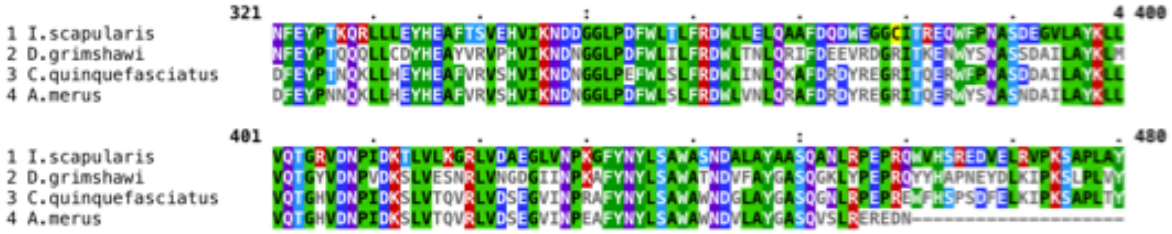


Figure S4. Sequence alignment of a part of Patched superfamily domain. Primary sequence alignment of the Patched from Ixodes scapularis (EEC01563.1), Drosophila grimshawi (XP_001987227.2), Culex quinquefasciatus (XP_038109984.1), and Anopheles merus (XP_041782449.1). Patched from Drosophila grimshawi has 1106 amino acids long Patched superfamily domain, its part is in alignment. The sequence alignment was generated using Clustal Omega.